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Glyscavins A, B and C, New Phenolic Glycoside Antioxidants Produced by a Fungus *Mycelia sterilia* F020054

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Abstract Three new phenolic glycosides designated glyscavins A (**1**), B (**2**), and C (**3**) were isolated from the culture broth of a fungal strain *Mycelia sterilia* F020054. Structural elucidation of the compounds was based on the NMR and MS spectroscopic analyses. Glyscavins A, B and C exhibited higher free radical scavenging activity on superoxide and 2,2′-azinobis(3-ethylbenzothialozinesulfonic acid) cation radical ($ABTS^{\cdot+}$) than butylated hydroxyanisole (BHA).

Keywords glyscavin, phenolic glycosides, *Mycelia sterilia*, antioxidant

Free radicals abstract hydrogen atoms from biological molecules, which is directly responsible for the detrimental effects of oxidative stress. Free radicals induce oxidative damage of cellular lipids, nucleic acids and proteins, are thought be one of major risks for cancer, atherosclerosis, diabetes mellitus, coronary heart disease, inflammation, cerebral ischemia, skin damages, and various other degenerative diseases [1∼4]. Thus, free radical-scavenging antioxidants have the potential as protective agents against various diseases caused by oxidative damage [5, 6]. We have searched for new biologically active substances from various natural resources [7∼10]. As a part of our continuing search for new free radical scavengers as skin protecting agents, we investigated the *n*-BuOH-soluble portion of the fermentation broth of the fungal strain F020054. Activity guided fractionation of the *n*-BuOH extract led to the isolation of three new phenolic glycosides designated glyscavins A (**1**), B (**2**), and C (**3**). This paper describes the isolation and structural elucidation of **1**∼**3**, primarily by extensive NMR experiments. The antioxidant activities of **1**∼**3** evaluated by using the free radicals such as superoxide and $ABTS^{\cdot+}$ are also described.

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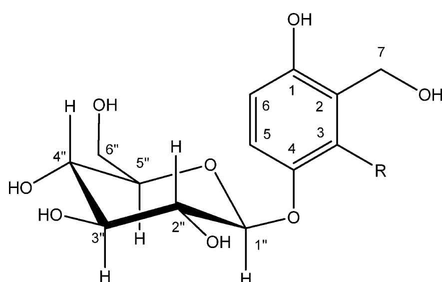
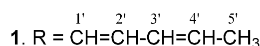
Results and Discussion

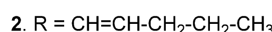
Glyscavins A (**1**), B (**2**), and C (**3**) were obtained as yellow amorphous powders. Acid hydrolysis of **1**∼**3** gave D-glucose, which was confirmed by co-TLC with authentic sample and NMR data. The molecular formula of **1** was determined as $C_{18}H_{24}O_8$ by HRFAB-MS [$(M+Na)^+$, *m/z* 391.1367; calcd: 391.1369] in combination with ^{13}C NMR data. In the IR spectrum, **1** exhibited strong hydroxyl band at ν_{max} 3402 cm^{-1}, whereas its UV spectrum showed maxima at 223, 271, and 388 nm. The ^{13}C NMR data (Table 1) depicted 18 carbon signals distributed into 1,2,3,4-tetra substituted benzene ring, four olefinic methines, an isolated methylene, one methyl group, and β-glucopyranosyl group. In the ^1H NMR spectrum of **1**, two aromatic signals were assigned to H-5 (δ 7.02, d, J=9.0 Hz) and H-6 (δ 6.65, d, J=9.0 Hz), which suggested the existence of 1,2,3,4-tetra substituted benzene ring. In addition, four olefinic protons at δ 6.71, 6.75, 6.27 and 5.81, and β-linked glucoside at δ 4.76 (1H, d, J=7.2 Hz), 3.85 (1H, dd, J=12.0, 2.4 Hz), 3.65 (1H, dd, J=12.0, 5.4 Hz), and 3.45∼3.33 (4H, m) were observed. The coupling constant of an anomeric proton at δ

4.76 (d, J=7.2 Hz) indicated that the glycosyl linkage is β-configuration. The proton spin system, together with the chemical shift values of the related carbons [δ 103.8 (C-1″), 75.1 (C-2″), 78.3 (C-3″), 71.4 (C-4″), 78.1 (C-5″) and 62.6 (C-6″)] were typical of a glucose moiety.

The protons that were directly attached to carbons were assigned with the aid of HMQC spectrum. The HMBC correlations between δ 6.71 (H-1′) and δ 125.8 (C-2), 149.9 (C-4) showed the connection of the C_5 unit containing olefinic protons with the 1,2,3,4-tetrasubstituted benzene ring. The long-range couplings observed from H-7 to δ 131.3 (C-3) and 153.3 (C-1) in the aglycon place the hydroxy methyl group on C-2. The presence of penta-1,3-dienyl moiety was verified by the analysis of ^{1}H-^{1}H COSY and HMBC data, and in turn this group was connected to C-3 on the basis of HMBC correlation of H-2′ with C-3. The glucopyranosyl group connected to C-4 (δ 149.9) through an *O*-glycosydic bond, as was confirmed by the downfield chemical shift of C-4 and the HMBC correlations between the anomeric proton [δ 4.76 (H-1″)]. The regiochemistry of C-1′ and C-3′ was determined as trans by proton coupling constant of 15.6 and 15.0 Hz, respectively. The overall structural determination of **1** was based on the detailed analysis of 1D and 2D NMR spectroscopic data including the ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and HMBC spectra. On the basis of aforementioned evidence, the structure of **1** was assigned as 4-*O*-β-D-glucopyranosyl-2-hydroxymethyl-3-penta-1,3-dienyl)phenol.







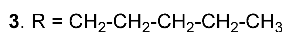


Fig. 1 Structures of glyscavins A (**1**), B (**2**), and C (**3**).

The molecular formula of glyscavin B (**2**) was

Table 1 ^{1}H (600 MHz) and ^{13}C NMR ^{1}H (150 MHz) data for compounds **1**, **2** and **3** in CD_3OD (δ; J in Hz)

Position	**1**		**2**		**3**	
	^{13}C^a	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	153.3		153.1		152.8	
2	125.8		125.8		126.5	
3	131.3		131.4		134.5	
4	149.9		149.5		150.4	
5	118.3	7.02, d (9.0)	118.2	7.00, d (9.0)	117.9	7.00, d (9.0)
6	114.9	6.65, d (9.0)	114.7	6.65, d (9.0)	113.9	6.60, d (9.0)
7	58.2	4.72, d (13.5)	58.6	4.72, s	57.5	4.71, d (12.6)
		4.70, d (13.5)				4.69, d (12.6)
1′	124.9	6.71, d (15.6)	124.6	6.59, brt (15.9)	27.1	2.79, m
2′	136.8	6.75, dd (15.6, 8.7)	138.2	6.04, dt (15.9, 6.9)	31.7	1.52, m
3′	134.2	6.27, ddd (15.0, 8.7, 1.2)	36.8	2.23, brq (6.6)	33.3	1.37, m
4′	130.5	5.81, dd (15.0, 6.6)	23.6	1.53, m	23.6	1.37, m
5′	18.4	1.80, dd (6.6, 1.2)	14.1	0.99, t (6.0)	14.5	0.91, t (6.6)
1″	103.8	4.76, d (7.2)	103.6	4.77, d (7.2)	104.1	4.72, d (7.2)
2″	75.1	3.45, brt (8.4)	75.0	3.45, brt (8.4)	75.2	3.45, brt (8.4)
3″	78.3	3.42, t (9.0)	78.1	3.43, t (9.0)	78.5	3.42, t (9.0)
4″	71.4	3.37, t (9.0)	71.3	3.38, t (9.0)	71.5	3.37, t (9.0)
5″	78.1	3.33, m	77.8	3.30, m	78.0	3.34, m
6″	62.6	3.65, dd (12.0, 5.4)	62.5	3.69, dd (12.0, 5.4)	62.7	3.69, dd (12.0, 5.4)
		3.85, dd (12.0, 2.4)		3.85, dd (12.0, 2.4)		3.87, dd (12.0, 2.4)

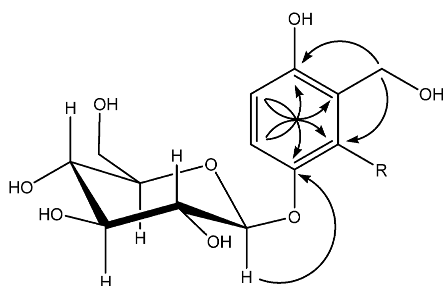


Fig. 2 Significant HMBC correlations for glyscavins.

Table 2 Free radical scavenging activity of compounds **1**~**3**[a]

Compound	Superoxide	ABTS$^{\cdot+}$
1	1.17	1.90
2	1.19	2.11
3	3.63	2.42
BHA	6.00	11.28

[a] Results are expressed as EC_{50} (μM) to scavenge 50% free radical.

determined to be $C_{18}H_{26}O_8$ on the basis of high resolution FAB-MS [$(M+Na)^+$, *m/z* 393.1522; calcd: 393.1525]. The ^{1}H NMR and ^{13}C NMR data as well as 2D correlation spectra suggested that both of the functional groups and the general substitution patterns present in **2** were similar to those of **1**. The ^{1}H and ^{13}C NMR spectra of **2** also resembled those of **1** except for the presence of two additional methylenes (C-3′ and C-4′) instead of two olefinic methines in **1**. The structure of **2** was assigned by the HMBC experiment in combination with the ^{1}H-^{1}H COSY and HMQC spectra. The HMBC showed the critical correlations from δ 2.23 (H-3′) to δ 124.6 (C-1′) and 14.1 (C-5′), from δ 1.53 (H-4′) to δ 138.2 (C-2′) and 14.1 (C-5′), establishing the structure of **2** as 4-*O*-β-D-glucopyranosyl-2-hydroxymethyl-3-pent-1-enyl)phenol.

The molecular formula of **3** was determined to be $C_{18}H_{28}O_8$ on the basis of HRFAB-MS [$(M+Na)^+$, 395.1680 *m/z*; calcd: 395.1682]. The NMR data of **3** showed that the aromatic units and glucose moiety were almost identical to those of **2**. Significant difference in the ^{1}H NMR spectra between **3** and **2** were the absence of the signals for the olefinic protons at δ 6.59 (H-1′) and δ 6.04 (H-2′) in **2**, and the appearance of the signals for two methylene protons at δ 2.79 (H-1′) and 1.52 (H-2′) in **3**. This was confirmed by observing the methylene carbons at δ 27.1 (C-1′) and 31.7 (C-2′) in the ^{13}C NMR spectrum of **3**. The HMBC spectrum showed the critical correlations from δ 2.79 (H-1′) to δ 126.5 (C-2), 150.4 (C-4) and 33.3 (C-3′), from δ 1.52 (H-2′) to δ 134.5 (C-3) and 23.6 (C-4′), establishing the structure of **3** as 4-*O*-β-D-glucopyranosyl-2-hydroxymethyl-3-pentylphenol.

Antioxidant activity of **1**~**3** was evaluated by using superoxide and ABTS$^{\cdot+}$ radical scavenging activity of **1**~**3** (Table 2). Compounds **1**~**3** exhibited superoxide radical scavenging activity, in a dose-dependent fashion, with EC_{50} values of 1.17, 1.19 and 3.63 μM, respectively. It also scavenged ABTS$^{\cdot+}$ with EC_{50} values of 1.90, 2.11 and 2.42 μM, respectively. Compounds **1**~**3** showed stronger free radical scavenging activity than BHA used as a standard antioxidant.

Experimental

General

Optical rotations were measured at 25°C using a JASCO DIP-370 polarimeter. The UV spectra were recorded on a Shimadzu UV-260 spectrophotometer. FT-infrared spectroscopy was done on a FT-IR Equinox 55 spectrometer using KBr pellets. ^{1}H and ^{13}C NMR spectra and 2D NMR experiments were recorded in CD_3OD on a Bruker DMX 600. Chemical shifts of ^{1}H and ^{13}C NMR are given in δ values (ppm) referenced to the methyl group of CD_3OD at 3.30 and 49.9 ppm, respectively, as an internal standard. ESIMS data were obtained using a JEOL JMS-SX 102A instrument at 70 eV. HRFAB-MS spectra were measured on a JEOL JMS HX-110 mass spectrometer with matrix of triethanolamine. Open column chromatography was carried out on silica gel 60 (70~230 mesh, Merck) and Sephadex LH-20 (Pharmacia Co. Ltd.). Analytical TLC was performed with Kiesel gel 60 F_{254} (Merck) without activation. HPLC was carried out with a Waters HPLC instrument equipped with a Waters 996 photodiode array detector set at 200~600 nm and a Waters 515 pump, using a C_{18} (YMC J'spherel ODS-H80 150×20 mm). Control of the equipment, data acquisition, processing, and management of chromatographic information were performed by Waters Millennium 2000 software program.

Organism and Fermentation

The producing fungus, strain F020054, was isolated from a soil sample collected at Nodong cave in Chungbuk province, Korea. The taxonomic study of the strain was conducted by the staff of Korea Research Institute of Bioscience and Biotechnology, Korea. The strain showed very low homology with reported fungal organisms, and did not form spores. Thus, the strain was tentatively named as *Mycelia sterilia* F0200054. The strain was deposited at

the Korean Collection for Type Culture (KCTC), under accession No. KCTC 1089BP. For maintenance on agar slants and submerged cultures, the fungus was grown on PDA medium (potato dextrose broth 2.0%, agar 1.8%). For long-term maintenance, the strain was preserved in an YPS medium (glucose 2.0%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4$ 0.05%. KH_2PO_4 0.1%, pH 6.0) containing 15% glycerol at −70°C.

A loopful of the strain was inoculated from a PDA (Difco Co.) slant culture into 500-ml baffled Erlenmeyer flasks containing 120 ml of YPS medium. The flasks were shaken at 28°C on a rotary shaker at 140 rpm for 3 days. Ten ml of the seed culture was inoculated into 500-ml baffled Erlenmeyer flasks each containing 120 ml of the same culture medium. The fermentation was carried out at 28°C for 7 days on a rotary shaker at 140 rpm.

Extraction and Isolation

A total of 12 liters of the cultures was filtered to separate the broth filtrate and the mycelium cake. The broth filtrate was sequentially extracted with $CHCl_3$ and *n*-BuOH. The *n*-BuOH extract was concentrated *in vacuo* and then was subjected to a silica gel column (Merck silica gel 60), eluted with $CHCl_3$ - MeOH mixtures of increasing polarities (50 : 1 to 3 : 1), to obtain three active fractions. The active fractions were concentrated and rechromatographed on a silica gel column eluted with $CHCl_3$ - MeOH (20 : 1) to afford active fractions. The active fractions were evaporated and subjected to Sephadex LH-20 column chromatography eluted with MeOH. An active fraction was purified by reversed-phase preparative HPLC (YMC J'sphere ODS-H80, 150×20 mm) developed with 40% aqueous MeOH at a flow rate of 6.0 ml/minute. The peaks with a retention time of 36, 46 and 56 minutes were collected and concentrated to give dark yellow powdery materials of **1** (8.7 mg), **2** (15.2 mg), and **3** (159.6 mg).

Superoxide Anion Radical Scavenging Activity

Superoxide radical scavenging activity was measured by employing a modified method of Beauchamp and Fridovich [11] as described by Kim [12]. The mixture consisted of 140 μl of 0.030 mM riboflavin, 1.0 mM EDTA, 0.60 mM methionine and 0.030 mM NBT solution in 50 mM potassium phosphate buffer (pH 7.8) and 10 μl of a sample solution, which includes the test compounds and the reference compound at various concentrations in DMSO, as well as DMSO as a control. The photoinduced reactions to generate superoxide anion were carried out in an aluminum foil-lined box with two 20 W fluorescent lamps. The distance between reactant and lamp was adjusted until the intensity of illumination reached 1000 lux. The reactant was illuminated at 25°C for 8 minutes. The photochemically reduced riboflavin generated superoxide anion, which reduced NBT to form the blue formazan. The un-illuminated reaction mixture was used as a blank. Reduction of NBT was measured by the absorbance change at 560 nm before and after irradiation using a microplate. Scavenging activity was calculated from the absorbance changes of control and test samples:

$$\text{Scavenging activity (\%)} = (1 - \Delta A_{sample}/\Delta A_{control}) \times 100$$

where ΔA_{sample} is the change of the absorbance in the wells containing the tested compounds, and $\Delta A_{control}$ is the change of the absorbance in the wells containing DMSO instead of the tested compounds.

The EC_{50} value is defined as the concentration of substrate that causes 50% loss of the reduced NBT. The assays were performed in triplicate and the absorbance changes were averaged before calculation.

ABTS Cation Radical Scavenging Activity

The total antioxidant activity of the compounds was determined using the $ABTS^{\cdot+}$ scavenging assay of Re [13]. The ABTS cation radical was produced by the reaction between 7.0 mM ABTS and 2.45 mM potassium persulfate in water for 12 hours in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with PBS until $A_{734}=0.7$. The reaction was initiated by adding 190 μl of $ABTS^{\cdot+}$ to 10 μl sample solution at 25°C. The percentage of reduction of A_{734} was recorded and was plotted as a function of the sample's concentration.

Compound **1**: yellow powder; HRFAB-MS $[M+Na]^+$ *m/z* 391.1369 (calcd for $C_{18}H_{24}NaO_8$, 391.1369); $[\alpha]_D^{25}$ −21.9° (*c* 0.2, MeOH); IR ν_{max} 3402, 2925, 2650, 1377, 1244, 1075 cm^{-1}. UV (MeOH) λ_{max} nm (log ε) 223 (4.13), 271 (4.12), 388 (3.74); ^1H and ^{13}C NMR, see Table 1.

Compound **2**: yellow powder; HRFAB-MS $[M+Na]^+$ *m/z* 393.1522 (calcd for $C_{18}H_{26}NaO_8$, 393.1525); $[\alpha]_D^{25}$ −110.7° (*c* 0.5, MeOH); IR ν_{max} 3394, 2928, 1707, 1394, 1244, 1076 cm^{-1}. UV (MeOH) λ_{max} nm (log ε) 225 (3.75), 299 (3.12); ^1H and ^{13}C NMR, see Table 1.

Compound **3**: yellow powder; HRFAB-MS $[M+Na]^+$ *m/z* 395.1680 (calcd for $C_{18}H_{28}NaO_8$, 395.1682); $[\alpha]_D^{25}$ −12.8° (*c* 0.2, MeOH); IR ν_{max} 3368, 2928, 1657, 1359, 1243, 1077 cm^{-1}. UV nm (MeOH) λ_{max} nm (log ε): 223 (3.53), 367 (3.35); ^1H and ^{13}C NMR, see Table 1.

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